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CONTENTS

SEC. C.—BOTANICAL SCIENCES

Page

Investigations Relative to the Breeding of Coumarin-free Sweet Clover, *Melilotus*—*T. M. Stevenson and J. S. Clayton* 153

A Four-year Quantitative Study of Nitrogen-fixing Bacteria in Soils of Different Fertilizer Treatment—*A. G. Lochhead and R. H. Thexton* - - - - - - - - - - - - - 166

Thermal Death Point of Certain Weed Seeds—*Clarence Yardley Hopkins* - - - - - - - - - - - - - - - 178

SEC. D.—ZOOLOGICAL SCIENCES

Studies on the Endoparasitic Fauna of Trinidad. III. Some Parasites of Trinidad Carnivores—*Thomas W. M. Cameron* - 25

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INVESTIGATIONS RELATIVE TO THE BREEDING OF COUMARIN-FREE SWEET CLOVER, *MELILOTUS*[1]

BY T. M. STEVENSON[2] AND J. S. CLAYTON[3]

Abstract

A series of studies was made upon the coumarin content of various species, varieties and individual plants of sweet clover *(Melilotus)*, determinations being made from different parts of the plant, at different stages of growth, and upon herbage dried by different methods. The conclusions drawn from the results of these studies may be summarized as follows:— (1) Coumarin content of the leaf and stem of sweet clover changes rapidly throughout the various stages of growth. (2) Wide variations in coumarin content exist between different species, between different varieties within a species and often between different individuals within a variety. (3) There is a marked relation between color of leaf and coumarin content. Plants possessing dark-colored foliage have invariably tested higher in coumarin content than those with foliage of lighter color. (4) The Alpha variety possesses a lower coumarin content than any other variety of *M. albus* tested. (5) The species, *Melilotus dentatus*, used in these tests, contained less than 0.01% of coumarin in the foliage and less than 0.05% in the mature seeds. For all practical purposes it may be regarded as coumarin-free. (6) Air drying or oven drying of sweet clover results in a heavy loss of coumarin from the leaves and marked changes in the coumarin content of the stems. (7) There is a definite correlation between the coumarin content of the leaf and of the mature seed in the materials used. (8) The coumarin content of the mature seed provides a reliable estimate of the coumarin content of the leaf of the plant. (9) Breeding results indicate the possibility of producing low coumarin varieties through inbreeding and selection.

Introduction

The investigations discussed in this paper were begun in 1933 and have been continued to the present time. The original purpose of the work was to ascertain facts which might be of assistance in selecting material for use in breeding coumarin-free sweet clover *(Melilotus)* of agricultural value. While, so far as the writers have been able to learn, no exact data are available with respect to the relative values of bitter and non-bitter sweet clover for hay and pasture purposes, there can be no doubt that the elimination of the bitter principle improves palatability. In addition, the possibility of coumarin and related compounds being in some manner associated with the toxic properties of sweet clover has not, as yet, been entirely eliminated. The production of coumarin-free varieties will make it possible to deal with this question more effectively.

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The fact that certain species of *Melilotus* are relatively free from coumarin has been recognized for some time. Kuznetsov (7) describes *M. dentatus* as being almost entirely free from the odor of coumarin, or as containing very small amounts of this substance. Unfortunately, however, this species possesses certain other characters which make it relatively undesirable as a cultivated plant. It therefore seems desirable to seek non-bitter types in species of greater agricultural importance.

In this study an effort has been made to determine: (a) the relative proportions of coumarin present in the stems and leaves of sweet clover at various stages from early growth to maturity, (b) what correlation, if any, exists between the amount of coumarin present in the leaves or stems and the mature seeds of the same plant, (c) the variation in coumarin content between different species, different varieties and different individuals within a species, variety or strain, (d) the effect of air drying or oven drying upon coumarin content, (e) the stage at which parent breeding material may be best selected.

Obermayer (8) was the first to develop a quantitative chemical test for coumarin in sweet clover. As a practical method for plant selection work, this test has been criticized by Ufer (10) on the grounds that it is not adapted to large-scale determinations.

Ufer (10) devised a qualitative test for coumarin in sweet clover, by means of which he has been able to test a large number of individual plants in a comparatively short time. Details of the test are not available. His selections were based upon the coumarin content of the first leaves of the sweet clover seedlings, which, he claims, possess a higher coumarin content than later leaves. He found, however, that some plants which showed a very low coumarin content in the first year showed appreciable amounts in the second year of growth. His results show the general trend of coumarin content in biennial sweet clover plants to be low in early growth stages of the first year, high in the fall of the first year, low in early growth stages of the second year and high in mature stages. The individuals that showed a relatively low coumarin content in the qualitative test were later tested quantitatively, by a modification of the Obermayer method. Coumarin-free selections from *M. albus* and *M. dentatus* were selected but failed to flower and died in the second year.

Kanevskaja and Fedrova (5) developed a quantitative test for the determination of coumarin and melilotic acid, based upon a differential ether extraction and subsequent gravimetric determination of both constituents. They report the results of analyses made on the leaves and on the stems of sweet clover plants.

Suvorov (9) states that a number of selections have been made from *M. albus*, *M. officinalis*, *M. wolgicus* and *M. dentatus*, which show as little as 0.05% coumarin. He also states that the Alpha variety showed a coumarin content of only 0.44%, as compared with 1.0% for the common type of biennial white blossom sweet clover.

Chelchinskaya and Bordunova (1) report results of investigations into the coumarin content of sweet clover, using a qualitative test as a basis of selection, while a modified form of Obermayer's procedure was used for quantitative determinations on air-dried material. They found the coumarin content of the stem to be markedly lower than that of the leaf and the coumarin content of both stem and leaf to vary greatly at different stages of growth, being highest at the flowering stage, and after this decreasing to a minimum at full maturity.

Duncan and Dustman (4) suggest a modification of Obermayer's procedure, using a steam distillation method of plant extraction for sorting out sweet clover plants of various coumarin contents.

Kirk (6) compared various types of sweet clover with respect to coumarin content, using Obermayer's method. The samples were taken at time of cutting for hay and were dried to constant weight in the laboratory before analyses were made.

Clayton (2) devised a qualitative color test for coumarin and melilotic acid in sweet clover. This test is based upon the coupling of coumarin with diazotized *p*-nitraniline in alkaline solution to give a crimson coloration.

Clayton and Larmour (3) have shown this test to be capable of quantitative interpretation with pure coumarin and melilotic acid solutions, and that a comparative, if not absolutely quantitative, determination of coumarin could be made upon plant extracts.

Experimental Procedure

The requirements of the colorimetric method of coumarin determination (Clayton and Larmour (3)) which has been used throughout these investigations are such that only a small amount of material is needed for testing. Thus uniformity to avoid errors in sampling is essential. During the earlier stages of the work one or two complete stems were taken, for each test, from individual plants. The same plants were used throughout the season. While this procedure insured that any variations in coumarin content obtained would be due to differences in stage of growth as between different times of sampling, it soon became evident that the constant pruning of the plants encouraged second growth and consequently the plants did not represent the normal growth stages. In view of this difficulty it was found necessary to use different individuals for each test. This, of course, raises the question of variation in coumarin content between different individuals within the same variety or strain. Results obtained indicate that differences do occur between individuals in certain varieties, but tests indicated a remarkable uniformity, at any particular stage of growth, among individuals within the several inbred lines used.

Preliminary tests indicated that a heavy loss in coumarin resulted from oven drying or air drying green plant material. It was decided therefore, in order to obtain the most accurate estimate of the natural coumarin content,

that the plant material should be tested in the fresh, green condition. Moisture determinations were made for each sampling and the coumarin content of each sample was expressed on the field-moisture basis. As far as possible, tests were made in duplicate. Sampling of the green material was done early in the mornings to reduce moisture loss to a minimum. No samples were taken when the plants were wet with rain or dew. Two entire stems were taken from each plant. One of these was used for moisture and the other for coumarin determination. Mature seeds were later harvested from the remaining stems of these individual plants and these, as well as a mass sample representing the entire variety or strain, were tested.

The leaves and stems of fresh green plants were examined separately. A 20-gm. sample, cut into thin sections, and 20 ml. of 50% methyl alcohol were put into a test tube marked at 50 ml. and fitted with rubber stopper and a 12-in. glass tube which served as an air condenser. The tube was immersed in a bath at 85° C. for 15 min. It was then removed, the sides were washed down with distilled water and the contents made up to 50 ml. with 50% methyl alcohol. A rubber stopper was inserted and the tube was then shaken for 1.5 hr. on a rotary shaker.

Seeds were extracted after first crushing them between layers of paper with a hammer and then grinding them in a mortar. A 0.5-gm. sample was put into the test tube, 50% methyl alcohol was added to the mark, the tube was stoppered and allowed to stand at room temperature for 12 to 16 hr. with occasional shaking, and was then shaken for 1 hr. on the rotary shaker.

In making the test on the extract, large test tubes, graduated to hold 50 cc., have been found convenient. To 5 cc. of the extract is added 5 cc. of 1.1% sodium carbonate solution and enough distilled water to half fill the tube. The contents are shaken and heated to 80° C. in a water bath for 15 minutes. To each tube is then added 5 cc. of the diazonium solution* and the tube filled to the 50 cc. mark by adding distilled water. The tubes are then stoppered, shaken and allowed to stand for at least two hours before the color comparisons are made. The volume of extract used must not contain more than 0.001 gm. of coumarin in order to be comparable with the standards used for comparison.

In order to evaluate the amount of coumarin in the material extracted, it is necessary to compare the colors obtained with those given by known concentrations of coumarin. If the color produced by the coumarin was the only color present in the extract, it would be possible to make quantitative comparisons on the colorimeter with pure coumarin solutions. However, other materials in the plant extract interfere with an accurate quantitative comparison by this method. By making up solutions from non-coumarin-containing plant extract, such as alfalfa, to which a known amount of coumarin is added, it is possible to set up a series of standards which allow comparisons to be made with reasonable accuracy. Comparisons may be made in two

*Details regarding the preparation of the diazonium solution were given in a previous paper (3, p. 93).

ways. A series of standard solutions ranging from 0–1.0% coumarin can be made up and the color of the unknown compared with these under a white light. In this way an estimate of the coumarin content can be made as being between any two of the standards, for example between 0.3 and 0.35%; or the coumarin content of the unknown may be determined by selecting the standard which corresponds most closely. Determinations are checked in all cases by means of the colorimeter. Fresh color standards for purposes of comparison are made up every four weeks; these are prepared to cover a range from 0 to 1.20%.

When only qualitative tests are made, approximately two hundred samples of either leaf or mature seed can be run in a day. The number is very much less, approximately 25, when exact quantitative tests are required.

Materials Used

The materials used in these tests were taken from the field nursery and variety test plots at Saskatoon. Thirteen varieties of *M. albus*, five varieties of *M. officinalis*, three introductions of *M. suaveolens*, one introduction of *M. wolgicus* and one of *M. dentatus* were available. In addition a large number of inbred lines of biennial white blossom sweet clover, *M. albus*, were included.

In searching for low coumarin selections, tests were made on the mature seed of several thousand additional plants obtained from various sources. In 1933 several plants of relatively low coumarin content were selected, and the progenies grown from these were available for testing in 1935.

Experimental Data

Moisture Content

Moisture determinations were made on all leaf and stem material taken for coumarin determinations. Fig. 1 represents the trend in moisture content of samples taken throughout the growing season of the second year. In the early stages, prior to flowering, the moisture content of the stem is higher than that of the leaf but it falls rapidly, being much lower than that of the leaf during the period from flowering to seed setting. The moisture content of the leaf shows a similar trend but the fall is not so rapid and it does not reach as low a point before the leaves fall from the plant. During a short period (early flowering), leaf and stem are approximately equal in moisture content.

Considering the marked variation in moisture content of the leaf and stem throughout the growing season, it was decided to calculate the actual coumarin percentages obtained on the fresh green plant material to a standard moisture basis for purposes of comparison. Since the average moisture content of the entire plant, leaves and stem, is approximately 80% during early flowering or at the stage when it would normally be harvested for hay, it appeared desirable to calculate all coumarin percentages to a basis of 80% moisture. It is evident that to express the results on a 100% dry matter basis would result in fictitiously high values for the coumarin content in the green materials and the exaggeration of variations beyond their real value.

Coumarin Content of Dried Plant Material

Anyone familiar with the odor given off from sweet clover hay during the process of curing must realize that some coumarin is lost through volatilization. Exact data on the extent of this loss have not been available. During these tests an attempt was made to determine the effect of air drying, as in haymaking, and of oven drying upon the coumarin content of the leaf and stem. In Table I are presented the results obtained from two of the several species included in this test. The plant material was harvested in the very early blossom stage. Samples of the fresh green material were tested immediately after harvesting and samples of field-cured hay and of material dried in the oven at 175° F., for two hours, were also tested.

TABLE I

Comparison of coumarin content of green, air-dried and oven-dried sweet clover

(All results calculated to basis of 100% dry matter)

Species	Green material	Air-dried in field	Oven-dried
M. albus			
Leaf	1.53	0.75	0.46
Stem	0.33	0.79	0.03
M. officinalis			
Leaf	0.96	0.54	0.16
Stem	0.28	0.71	0.07

These data indicate that much of the coumarin is lost from the stem and leaf of oven-dried material. This is to be expected since coumarin is readily volatile at the temperatures used. The data obtained from the field-cured

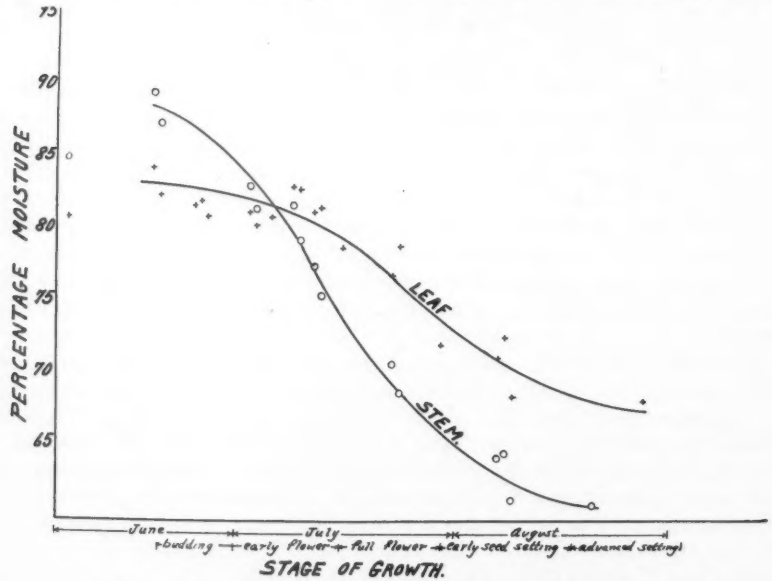

Fig. 1. *Moisture content of leaf and stem of sweet clover during the second year.*

samples is very interesting. On the dry-weight basis they show a distinct loss of coumarin from the leaves of the plant but also a marked accumulation of coumarin in the stems. This cannot be explained solely upon the basis of dehydration since the coumarin content is raised to a point beyond that shown by the stems of the fresh green material. It appears, therefore, that there is a translocation of coumarin to the stem from the leaf, or that coumarin is formed in the stem during the curing process. In view of these data it was decided that results more indicative of the actual coumarin content of the plant could be obtained by basing the test for coumarin upon the fresh, green, plant material than upon air-dried or oven-dried leaf and stem.

Variations in Coumarin Content During the Growing Season of the Second Year of Growth

The results of this phase of the work are based upon tests made, at various stages of growth, on ten lines of white blossom, biennial sweet clover which had been subjected to inbreeding and selection for at least three successive generations. This material was selected because of the apparent uniformity of type within the lines. Tests showed that there was relatively little variation in coumarin content between individuals within any of these inbred lines.

Tests for coumarin content were made on each of these lines at frequent intervals throughout the growing season. An effort was made to test samples from all lines at approximately the same stages of growth. The tests were started about thirty days prior to the time when the first buds became visible and were continued up to the time when the leaves fell and the plant matured seed. A single plant was taken from each line, for testing, at each stage of growth. Error due to individual variation, within a line, was reduced to a

TABLE II

Coumarin content (%) of leaf, stem and seed of 10 inbred lines of Arctic sweet clover (2nd year)

Plant No.	Stage of growth of vegetative material														Seed
	4-week to bud stage		2-week to bud stage		Bud stage		Early flowering		Medium flowering		Late flowering		Seed setting		Ripe seed
	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	Leaf	Stem	
1	0.23	0.06	0.20	0.12	0.17	0.07	0.08	0.02	0.11	0.04	0.03	0.01	–	0.01	0.40
2	0.34	0.16	0.19	0.10	0.18	0.07	0.08	0.02	0.15	0.02	0.02	0.01	–	0.01	0.40
3	0.43	0.09	0.19	0.12	0.16	0.05	0.12	0.03	0.15	0.02	0.04	0.01	–	0.01	0.47
4	0.30	0.09	0.19	0.10	0.21	0.07	0.14	0.02	0.17	0.02	0.09	0.01	–	0.01	0.66
5	0.25	0.05	0.26	0.10	0.19	0.05	0.12	0.02	0.12	0.02	0.09	0.01	–	0.01	0.57
6	0.29	0.22	0.23	0.11	0.17	0.05	0.21	0.03	0.17	0.02	0.08	0.02	–	0.01	0.45
7	0.34	0.17	0.23	0.10	0.28	0.07	0.28	0.04	0.25	0.06	0.14	0.02	–	0.02	0.47
8	0.36	0.16	0.17	0.11	0.18	0.07	0.20	0.03	0.17	0.02	0.06	0.01	–	0.02	0.43
9	0.22	0.07	0.14	–	0.18	0.05	0.14	0.02	0.15	0.02	0.03	0.01	–	0.01	0.39
10	0.19	0.07	0.13	0.03	0.16	0.06	0.08	0.02	0.13	0.02	0.07	0.01	–	0.01	0.46
Mean	0.30	0.11	0.19	0.09	0.19	0.06	0.15	0.03	0.16	0.03	0.07	0.01	–	0.01	0.47

minimum owing to the relatively high degree of uniformity within the various lines. All coumarin percentages obtained on the green material, both leaf and stem, were calculated to an 80% moisture basis. The coumarin content of the seed is given as actually determined.

In Table II are given the results obtained from each line and also the mean results for all of the lines included in the test. The general trend in coumarin content of the leaf and stem throughout the growing season is presented graphically in Fig. 2. From these data it may be observed that there is

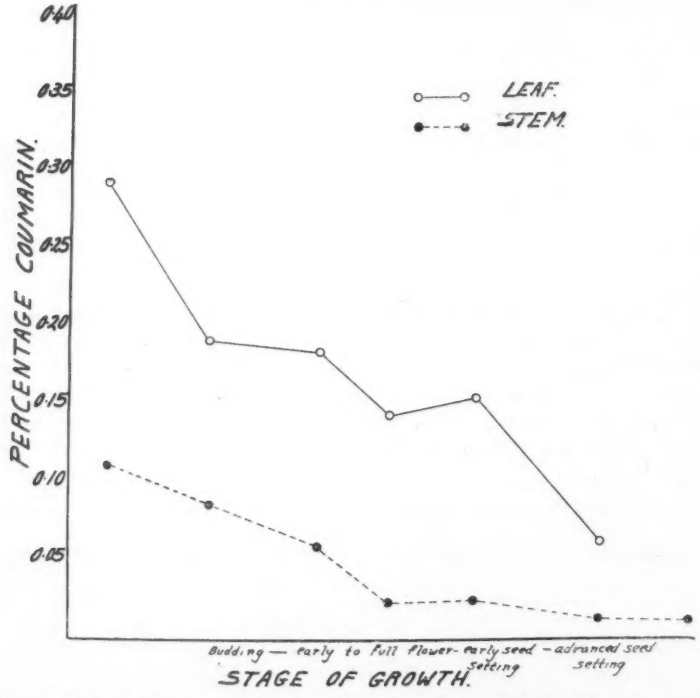

FIG. 2. *Mean coumarin content of leaf and stem of 10 inbred lines of biennial white blossom sweet clover M. albus.*

little variation between the lines with respect to the coumarin content of the stem material. They show a gradual falling off in coumarin content from before budding to the early flowering stage. From that point the fall is more rapid but there is a slight tendency to rise again at the early seed setting stage, which is again followed by a gradual decrease to an almost negligible amount at maturity. The coumarin content of the leaves shows greater variations between the different lines in that they do not all follow the same trend throughout the growing season. Fig. 3 shows the mean trend in coumarin

content of the leaf for all ten lines. Three distinct trends were obtained in the ten lines, however, and these are illustrated in Fig. 3. Type I shows a rapid falling in the coumarin content from four weeks prior to budding until

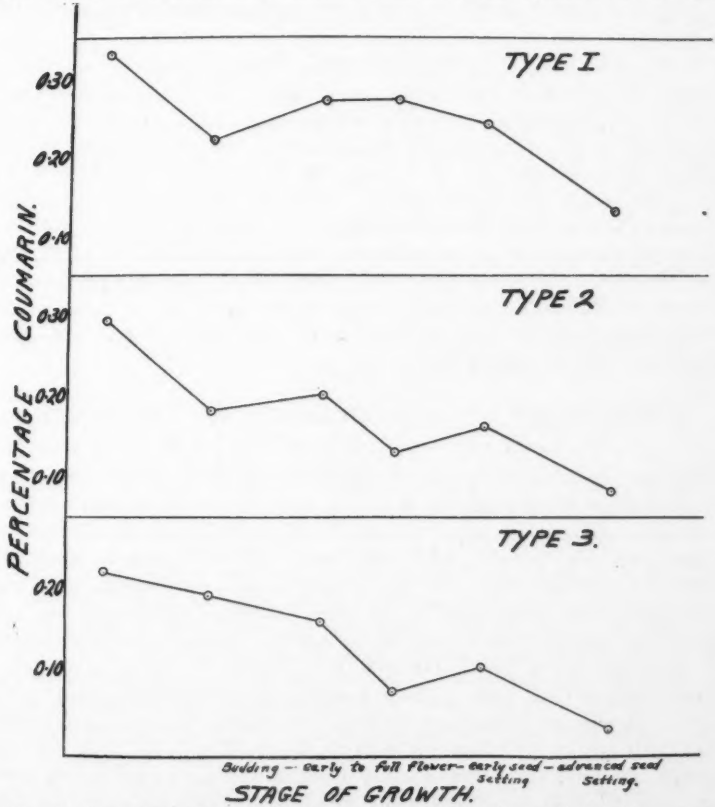

FIG. 3. *Coumarin content of leaf material of inbred lines of biennial white blossom sweet clover, M. albus.*

the budding stage. There is a gradual rise from budding to fall flowering and from this point a fairly rapid decrease to maturity. The rise in content during flowering does not reach as high a point as it attained in the early stage.

Type II shows a similar rapid falling off until the bud stage and a corresponding, although less pronounced, rise from bud to early flowering. This is followed by a very marked decrease during flowering and another increase during early seed setting, followed by a gradual decrease to maturity.

Type III shows a gradual decrease in coumarin to the early flower stage, then a rapid fall to full flower, followed by an increase during early seed-setting and finally a decrease from this point to maturity.

An analysis of these data discloses two tendencies common to all three types. First, the coumarin is higher during the period prior to budding than at any later stage. Secondly, after seed setting has begun there is a rapid falling off in coumarin content. The most common trend is represented by Type II.

It is of some interest that the rapid falling off in coumarin content during flowering corresponds to the period when the coumarin-like odor of sweet clover is most noticeable and also when the moisture content begins to diminish rapidly (See Fig. 1). It is possible that considerable coumarin is lost through volatilization, either directly or by being transformed into melilotic acid or melilotic anhydride, during this period. It should be noted that the changes in coumarin content are very great within short periods of time and that, consequently, variations in time of testing may lead to different results. No data were obtained before the plants had made eight or ten inches of growth in the spring of the second year, and consequently there are no data to show the trend during the period prior to 30 days before buds were visible. Such data will be obtained during 1936.

Comparison of Variations in Coumarin Content During the First and Second Years of Growth

Tests were made on the leaves of a number of varieties of different species, at various times throughout the first year of growth and on both the leaves and stems at different stages of growth in the second year. Seeds from this material were also tested. Tests were made on the first-year material as follows: the four-leaf stage, and 46, 74 and 94 days after planting. In the second year, tests were made at the early budding, early flowering, early seed-setting and advanced seed-setting stages. Data from some of these tests are presented in Tables III and IV.

Data obtained from tests made on material during the first year of growth show no significant differences between the various species and varieties tested. All appear to follow one general trend. Beginning with the young seedlings, four-leaf stage, the coumarin content in each case is low and in most cases it increases rather rapidly, reaching a maximum when the plants are about 10 to 12 in. high (in this case 46 days after the four-leaf stage), and then falls rather rapidly to the end of the growing season. Data obtained from tests made on the same material in the second year of growth reveal some rather striking differences between the various varieties and species used. The general trend, however, is a rise in coumarin content to a maximum during the flowering stage, followed by a steady decrease to maturity. In general the trend is similar to that obtained for the inbred lines of white blossom sweet clover. However, there were relatively few tests made on this material throughout the growing season, and consequently some of the variations in coumarin content, which take place rather rapidly at certain times, have not been recorded. The result is a tendency toward a unimodal curve with the highest point during the flowering period.

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TABLE III

COUMARIN CONTENT OF LEAF MATERIAL OF SOME *Melilotus* SPECIES (1ST YEAR).

Species	4-leaf stage	Days after planting		
		36	74	94
M. albus No. 1	0.48	0.45	0.24	0.22
M. albus No. 2	0.18	0.44	0.29	0.27
M. officinalis	0.37	0.51	0.23	0.20
M. suaveolens	0.24	0.44	0.30	0.27
M. dentatus (annual)	0.01	0.01	0.01	0.01*

**Less than 0.01 in each case.*

TABLE IV

COUMARIN CONTENT OF LEAF MATERIAL AND SEEDS OF SOME *Melilotus* SPECIES (2ND YEAR)

Species	Leaf material				
	Early budding	Early flowering	Flowering with seed-setting	Advanced seed-setting	Mature seed
M. albus No. 1	0.39	0.40	0.23	0.06	0.79
M. albus No. 2	0.18	0.23	0.15	0.09	0.37
M. officinalis	0.30	0.18	—	0.07	0.74
M. suaveolens	0.32	0.41	0.35	0.15	0.43
M. dentatus (annual)	0.01	0.01	0.01	0.01	0.05

The annual form of *Melilotus dentatus* included in these tests may be, for all practical purposes, regarded as coumarin-free. The tests made on the leaf and stem material show less than 0.01% coumarin and the mature seed 0.05%. It is worthy of note also that the coumarin content of the Alpha variety of biennial white blossom sweet clover is considerably lower than that of the other varieties of *M. albus* tested.

There appears to be no marked difference in coumarin content between the two most widely grown species, *M. albus* and *M. officinalis*. Variations between the different varieties within these species are fairly large.

During the testing of these various types and varieties of sweet clover it was noted that there appeared to be a marked relation between the coumarin content and the color of the foliage. Sweet clover presents marked variations in color of foliage, which ranges from light yellowish green to dark, almost bluish, green. The plants with dark colored foliage invariably contained a higher coumarin content than those possessing the lighter green color.

COMPARISON OF COUMARIN CONTENT IN LEAF AND MATURE SEED

In view of the fact that rapid changes in the coumarin content of the leaf and stem take place during the growing season, it is essential that tests be conducted at the same stage of growth for all individuals, if comparative data of value are to be obtained. It appears desirable therefore to seek some phase

in the life history of the plant, in which the coumarin content is more constant, upon which to base coumarin determinations. Thus an effort was made to determine to what extent, if any, the coumarin content of the leaf is reflected in that of the mature seed. The coumarin content of the leaf at full flowering stage, correlated with that of the mature seed for the twenty different varieties and species tested, showed a fairly significant correlation.

Breeding Work

In the winter of 1933, seeds from several thousand single plant selections were tested for coumarin content. Five selections were made which contain between 0.10 and 0.15% coumarin. Four individuals contained more than 1.0%. The average for all plants tested was 0.57%. Progenies from the selections of lowest and highest coumarin content were planted in the field nursery in the spring of 1934. Selfed seed was harvested from the individual plants in these progenies during the fall of 1935 and has been tested for coumarin content. The frequency distribution of coumarin content of individuals is shown in Table V. The data from the low-coumarin selections

TABLE V

A COMPARISON OF COUMARIN CONTENTS OF PROGENIES GROWN FROM HIGH- AND FROM LOW-COUMARIN SELECTIONS OF WHITE BLOSSOM BIENNIAL SWEET CLOVER *(M. albus)*

Selection No.	Classes in percentage*																
	.05	.10	.15	.20	.30	.40	.50	.60	.70	.80	.90	1.0	1.05	1.10	1.2	1.3	1.4
1	–	–	–	12	50	20	1	–	–	–	–	–	–	–	–	–	–
2	–	–	1	15	32	25	5	4	–	–	–	–	–	–	–	–	–
3	–	–	11	26	24	7	2	1	–	–	–	–	–	–	–	–	–
4	–	–	–	5	17	12	7	2	–	–	–	–	–	–	–	–	–
5	–	–	3	11	21	3	–	–	–	–	–	–	–	–	–	–	–
6	–	–	–	–	–	–	–	1	2	6	4	14	4	3	6	3	–
7	–	–	–	–	–	–	1	–	2	2	–	4	1	3	14	5	–
8	–	–	–	–	–	–	–	–	1	1	2	2	3	5	11	6	2
9	–	–	–	–	–	2	10	5	8	3	3	1	1	–	–	–	–

NOTE:—*The low-coumarin selections, Nos. 1–5 inclusive, contained less than 0.15% coumarin. The high-coumarin selections, Nos. 6–9 inclusive, contained a coumarin content of 1% or higher.*

**Class figures given represent upper limits of the class range.*

show definitely that most of the population tend to be relatively low in coumarin, an appreciably large percentage of the individuals showing a coumarin content of between 0.1 and 0.2%. The data from the high-coumarin selections, on the other hand, show that the greater part of the populations are of relatively high coumarin content. The greatest frequencies are in the 0.91–1.2% coumarin classes. The coumarin content of both low- and high-coumarin progenies tends to be higher than that of the corresponding original selections which were grown the previous year. This is believed to be due to seasonal influences.

The progeny plants from the low-coumarin lines appeared perfectly normal in the field. There were no indications of lack of vigor. Without exception, these plants produced seed abundantly.

These results indicate that the coumarin content of *M. albus* is determined largely by heredity, and that it may be possible to isolate lines containing relatively little coumarin, through continuous selection within inbred lines.

The other phase of the breeding work has to do with crossing the almost coumarin-free *M. dentatus* with species of recognized agricultural value. Up to the present time we have not been successful in bringing to maturity the plants presumed to be F_1 hybrids. Seeds were obtained without difficulty when *M. albus*, as the female parent, was crossed with *M. dentatus*. These seeds germinated but the young seedlings were, without exception, weak and lacking in chlorophyll. They died within two or three weeks after emergence. No seeds were obtained from the reciprocal cross.

Acknowledgments

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A FOUR-YEAR QUANTITATIVE STUDY OF NITROGEN-FIXING BACTERIA IN SOILS OF DIFFERENT FERTILIZER TREATMENT[1]

By A. G. Lochhead[2] and R. H. Thexton[3]

Abstract

Numbers of *Rhizobium trifolii*, *Rh. leguminosarum*, *Rh. meliloti* and *Azotobacter* were determined at four-week intervals throughout a four-year crop rotation in three soils which had been receiving for twenty years no fertilizer, manure, and artificial fertilizer respectively. Though relatively small differences were noted in numbers of *Rh. trifolii* in the three soils, *Rh. leguminosarum* and *Rh. meliloti* persisted in much higher numbers in the two fertilized areas than in the unfertilized soil. *Rh. trifolii*, the only species with host plant in the rotation, occurred in much greater numbers than the other species, not only during and immediately following clover, but in succeeding years when little or no decline was noted. Apart from the effect of clover on *Rh. trifolii* no significant effect of cropping was noted nor was seasonal influence important. Freezing of the soil for three months each year produced little or no effect on the numbers of *Rhizobia*.

Numbers of *Azotobacter* were consistently higher in the unfertilized soil than in the fertilized areas. A seasonal effect was noted, with maximum numbers in March and minimum numbers in July, while freezing caused no noticeable diminution in colony count. The numbers of *Azotobacter* found were in all cases low and suggest that the part played by this organism in nitrogen fixation in field soil is still obscure.

Rh. trifolii, *Azotobacter* and total numbers of bacteria by the plate method showed no relation with the productivity of the soils. Numbers of *Rh. leguminosarum* and *Rh. meliloti* showed better agreement, though only in the case of *Rh. meliloti* were relative numbers consistent with the soils in order of crop yields throughout the rotation.

Introduction

The influence of such factors as season, cropping and fertilizer treatment upon the general microflora of soils has been the subject of considerable investigation. Less attention has been paid to the effect of season and soil management practices upon specific groups of soil micro-organisms. It might be reasonably assumed, however, that studies of the influence of various factors on the numbers and activities of definite physiological groups of soil micro-organisms would contribute more to our knowledge of soil processes than analogous data on the soil microflora as a whole. It is conceivable that pronounced changes in the numbers of two or more groups of organisms, reacting differently to a given influence, may be quite unrecognized by a method of "total counts."

The nitrogen-fixing bacteria, symbiotic and non-symbiotic, are commonly regarded as groups of organisms of definite significance in the maintenance of fertility in most arable soils. Being physiologically specialized types, they might be expected to react in their own fashion to the various influences of climate, cropping and soil treatment. The persistence of the different cross-inoculation groups of nodule-forming organisms in soil is of definite import-

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ance in relation to the growth of legume crops and the need for re-inoculation, while non-symbiotic fixation must be influenced by the numbers of nitrogen-fixing organisms as they react to their environment.

By far the greater number of quantitative studies of the microflora of soils have represented examinations carried out during the actual or potential crop producing period, that is, from spring to autumn. In regions such as are represented by the greater part of Canada, where the soil may remain frozen for a part of the year, often two to three months or more, it is reasonable to assume that the winter season must also be considered in attempting to form any comprehensive picture of soil microbiological processes as a whole. The present study is an attempt to follow for four consecutive years the numbers of three species of *Rhizobium* and of *Azotobacter* under definite conditions of cropping and fertilizer treatment.

Historical

Various studies of a qualitative nature have shown that certain species of nodule bacteria may remain alive in soils, apart from the host plant, for a number of years. Albrecht and Turk (1) in surveying the literature, emphasize the wide range in the period of time through which legume bacteria have been reported to survive. Little information of a quantitative nature respecting the incidence of *Rhizobium* in soils and the effect of environment on the numbers was available, however, until the development of a suitable method of enumeration. Through the application of a suitable dilution method, however, Wilson (20–24) was able to study quantitatively the legume bacteria population of soils, and indicate the value of a counting technique for studying the relations of different species of *Rhizobium* to their environment.

Examining a series of soils from October to June, Wilson (21) found in general a drop in numbers of *Rhizobium* as the winter advanced, followed by a rise in spring. Reporting studies (22, 24) on the relative numbers of different species of *Rhizobium*, he found that soils vary in their capacity to maintain different types. *Rh. trifolii* was found in general in much higher numbers than *Rh. leguminosarum* which latter species was in general more abundant than *Rh. japonicum*. Little or no significance was attached to the reaction of the soil, the moisture content, the season, or the crop on the soil. The author points out that the numerical relation of these species of *Rhizobium* in soils coincides with the hardiness of the organism in artificial cultures and also with the hardiness of the respective host plants in the field. From a special study (23) of *Rh. japonicum* the writer concluded that organisms liberated from the nodule tissue of the host crop diminish rapidly and reach minimum numbers or practical extinction in two or three years.

Walker and Brown (19) applied the Wilson method to a study of *Rh. trifolii* and *Rh. meliloti* in variously treated soils, and found that, in general, the numbers depended upon the previous cropping history of the land, and

upon fertilizer treatment. They found, however, that the condition of the soil with reference to organic matter, lime and phosphate, had a much greater influence on the numbers of organisms than the frequency of growth of the host plant. They suggest that recommendations for inoculation should be based, not only upon the cropping system, but also upon a knowledge of the soil management practices followed.

Azotobacter is an organism of wide distribution in soils. Its absence is probably due, in the majority of cases, to an unfavorable reaction, since it does not normally develop in soils of an acidity greater than pH 6.0, though Wilson and Wilson (25) point out the importance of the "soil complex," particularly the carbonate-phosphate ratio, in controlling the activity of this organism. Under laboratory conditions, response to additions of fertilizers to samples of soil has been repeatedly shown by this organism, with or without added inoculum; and the development of the soil plaque method has added to our methods of estimating fertilizer requirements. There is, however, much less information as to the numbers of this organism in soil under field conditions, and to the effect of climatic and soil management practices.

Quantitative studies have indicated that *Azotobacter* is present in soils in comparatively small numbers, generally hundreds or a few thousands per gram, though microscopic studies would indicate larger numbers (18). Our information as to the effect of environment on *Azotobacter* in the soil has been largely inference from nitrogen-fixation tests carried out in soil or solution. Since nitrogen fixation in soils is by no means the exclusive function of *Azotobacter* the occurrence of this organism is more accurately measured by more direct methods. Evidence of a more direct nature was possible by the use of the silica-gel plating medium devised by Winogradsky (26) for developing colonies of *Azotobacter* directly from soil.

Application of the silica-gel plate method to the estimation of the *Azotobacter* population of soils has been made by a number of investigators (3, 11, 14, 16, 28, 29). Counts reported have been generally quite low, ranging for the most part from none to several hundred per gram of soil, with occasional counts showing several thousand per gram. Winogradsky and Ziemięcka (28) in a study of the effect of fertilizers, noted a depression of *Azotobacter* numbers in soils receiving applications of nitrogen. Curie (3), developing an agar plate method from the silica-gel technique, reported a lower *Azotobacter* population in soils receiving additions of manure, as compared with control plots, a similar depressing effect of nitrogen being noted by Ziemięcka (29) in a study of Rothamsted soils. Enumerating *Azotobacter* by the silica-gel plate method, Vandecaveye and Anderson (16) in a study of seasonal trend, found at most a few hundred colonies per gram in spring and fall, with organisms absent from gram portions in midsummer. Turk (14) likewise found small numbers in Michigan soils. In a recent study of Swiss soils, Stöckli (11) reported counts of a few hundreds or thousands, the numbers tending to rise with increase in pH value and amount of available phosphoric acid.

Methods

Determinations were made of the numbers of *Rhizobium trifolii*, *Rh. leguminosarum* and *Rh. meliloti* at four-week intervals from November, 1931, until October, 1935, in three soils of different fertilizer treatment supporting a rotation of mangels, oats, clover and timothy. The tests were commenced following the clover. At the start of the experiment the three soil areas had received for 20 years the following fertilizer treatments:

Soil N—No fertilizer.

Soil X—15 tons manure per acre, applied to mangels.

Soil Y—100 lb. nitrate of soda, 300 lb. superphosphate, 75 lb. muriate of potash to mangels, 100 lb. nitrate of soda to oats, clover and timothy.

The three soils, at the start, contained respectively 0.113%, 0.162% and 0.126% nitrogen, and all showed a reaction slightly more alkaline than pH 7.0. The productivity for 24 years and for the period of the experiment is indicated in Table I.

TABLE I

CROP YIELDS OF SOILS

—	Timothy, tons per acre	Mangels, tons per acre	Oats, bu. per acre	Clover, tons per acre
Soil N, av. 24 years	2.04	8.22	45.7	2.08
av. 1931-1935	1.82	2.15	47.1	1.49
Soil X, av. 24 years	3.11	22.46	59.9	3.79
av. 1931-1935	2.83	22.90	64.9	3.56
Soil Y, av. 24 years	2.67	20.74	54.6	3.45
av. 1931-1935	2.31	21.03	60.6	2.76

Samples were taken at a depth of two to three inches, normally from the sides of a pit approximately three feet square. When the soil was frozen it was sampled by removing about one square yard of the surface to a depth of two inches, with a pick, and chipping off portions from the two- to three-inch level. The samples, after thawing, if necessary, were passed through a 3-mm. sieve, mixed, and dilutions prepared by adding 20 gm. to 200 cc. sterile water.

The method of determining the numbers of *Rhizobium* was essentially that used by Wilson (20). Small enamel crocks of 250 cc. capacity containing a mixture of equal quantities of soil and sand were sterilized and inoculated in duplicate with 1 cc. of a series of dilutions (1–10, 1–50, 1–100, 1–500, 1–1,000, up to 1–10,000,000). After moistening with sterile water, the crocks were covered and incubated at 28° C. for one week. Seed of alfalfa, red clover and vetch, after being sterilized with mercuric chloride were planted in appropriate pots corresponding to the dilutions found necessary to insure absence of inoculation with the highest dilution. After two to three weeks in the greenhouse, depending upon the growth, the roots of the seedlings were examined for nodules, and the numbers of the three species of *Rhizobium* estimated from their capacity for nodule production on the respective host plants.

The numbers of *Azotobacter* were estimated by an adaptation of the agar plate method proposed by Curie (3). The medium used contained, per litre, mannite, 15.0 gm.; dipotassium hydrogen phosphate, 0.5 gm.; magnesium sulphate, 0.2 gm.; sodium chloride, 0.2 gm.; calcium carbonate, 1.0 gm.; manganese sulphate and ferric chloride, 1 drop of 5% solution each, and agar 1.5 gm. The medium was poured into ordinary Petri dishes (25 cc. each) and allowed to harden. Upon the surface was scattered uniformly 0.3 gm. of the soil, passed through a 20-mesh but retained on a 40-mesh sieve. For each sample of soil 10 plates were inoculated, incubated at 28° C. for three to four days, and *Azotobacter* colonies counted. Soils which were too moist to handle were partially air-dried before inoculating, moisture determinations being made on the soil so used as well as on the samples coming from the field. For the first year of the experiment *Azotobacter* counts were also made by the dilution method. Flasks of 200 cc. capacity, containing 25 cc. of a mannite solution similar to the above-mentioned medium, were inoculated, in duplicate, with decreasing quantities of soil. Flasks were incubated at 28° C. and examined microscopically for *Azotobacter*. During the first year, determinations were made of the so-called "nitrogen-fixing capacity" of the soils. Four 500 cc. Erlenmeyer flasks, containing 100 cc. of the above mannite solution, were inoculated with 5 gm. of soil. Two flasks were then sterilized as controls and two flasks incubated at 28° C. for 14 days, and total nitrogen determined by the usual Kjeldahl method.

During the last three years of the experiment determinations were made of total numbers of bacteria and actinomyces in the soils. Plate counts were made on the synthetic agar medium described by Thornton (12), incubated at 28° C. for 10 days.

Results and Discussion

The data from 55 consecutive analyses for the various organisms on the three soils, extending from November 10, 1931, until October 11, 1935, are shown graphically in Figs. 1 to 3, while yearly summaries (November to October) are given in Table II.

Rhizobium

The results indicate that the three species of *Rhizobium* studied not only varied in their actual abundance, but also showed relative differences varying with the soil. Thus in the case of *Rh. trifolii* (Fig. 1 and Table II) there is comparatively little difference in the capacity of the three soils to support this organism. In the first year following the crop of clover the manured area gave higher average counts, but in the following years less difference was noted between the soils. In the case of *Rh. leguminosarum* it is plain that Soil N (no fertilizer) supports a much lower population than either of the soils receiving fertilizer. Thus Soil X (manure) contained approximately 21 times as many, and Soil Y (artificial fertilizer) 25 times as many organisms as Soil N, considering averages for the four-year period. Similarly, with *Rh. meliloti* the two fertilized areas, X and Y, were found to support respectively 10 and 6 times as many organisms for the four-year period as Soil N.

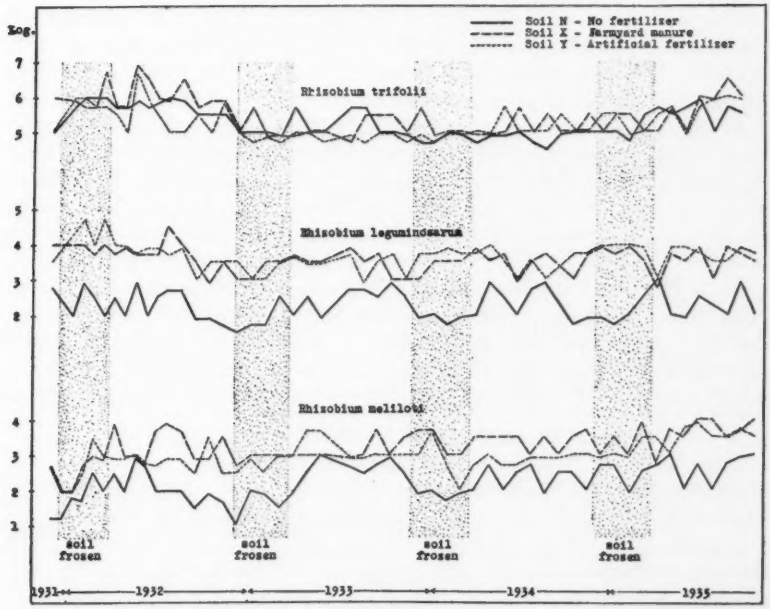

FIG. 1. *Numbers of Rhizobium trifolii, Rh. leguminosarum and Rh. meliloti in soils of different fertilizer treatment.*

TABLE II

SUMMARY OF COUNTS OF BACTERIA IN SOILS AT FOUR-WEEK INTERVALS FROM NOVEMBER, 1931 TO OCTOBER, 1935

Soil and year	Bacteria per gram of soil (log. average)				
	Rh. trifolii	*Rh. leguminosarum*	*Rh. meliloti*	*Azotobacter*	Total count
Soil N (No fertilizer)					
First year after clover	379,000	235	130	100	—
Second year after clover	161,000	155	155	113	13,700,000
Third year after clover	67,000	170	165	172	30,100,000
Clover crop	226,000	155	295	130	23,400,000
Average for 4 years	**174,000**	**175**	**176**	**123**	**21,500,000**
Average when soil frozen	**186,000**	**140**	**120**	**134**	**25,000,000**
Soil X (Manure)					
First year after clover	1,119,000	6,320	990	37	—
Second year after clover	158,000	2,690	1,160	61	22,400,000
Third year after clover	131,000	2,400	2,330	64	31,100,000
Clover crop	345,000	3,710	3,160	79	32,900,000
Average for 4 years	**310,000**	**3,600**	**1,690**	**55**	**28,300,000**
Average when soil frozen	**230,000**	**3,650**	**1,360**	**58**	**33,500,000**
Soil Y (Artificial fertilizer)					
First year after clover	370,000	7,360	590	59	—
Second year after clover	90,000	2,540	910	56	27,600,000
Third year after clover	104,000	3,360	720	67	33,400,000
Clover crop	293,000	5,370	2,810	76	31,200,000
Average for 4 years	**184,000**	**4,340**	**1,000**	**64**	**30,800,000**
Average when soil frozen	**156,000**	**5,890**	**890**	**69**	**39,200,000**

Rhizobium trifolii was present in all three soils in much greater numbers than the other legume bacteria studied. As it was the only species with its host plant in the crop rotation, its greater abundance during the year following clover might well be expected. After the first year further decline was slight or absent, however, the numbers remaining at a comparatively high level and suggesting that the three soils are capable of supporting a much higher population of *Rh. trifolii* than of *Rh. leguminosarum* or *Rh. meliloti*. This is in agreement with the results of Wilson (24), who reported finding *Rh. trifolii*, *Rh. leguminosarum* and *Rh. japonicum* in rather definite numerical order.

Table III shows the average relative numbers for the second and third years following clover, with comparative data calculated from Wilson's findings. In the fertilized areas, X and Y, ratios are of the same order as those reported by Wilson. Soil N, which had received no fertilizer for over 20 years, was relatively much less suited to *Rh. leguminosarum* and *Rh. meliloti*.

TABLE III

RELATIVE NUMBERS OF SPECIES OF *Rhizobium*

—	*Rh. trifolii*	*Rh. leguminosarum*	*Rh. meliloti*	*Rh. japonicum*
Soil N (no fertilizer)	1,000	1.6	1.6	—
Soil X (manure)	1,000	18	12	—
Soil Y (artificial)	1,000	30	8	—
Wilson (average of 55 samples)	1,000	14	—	0.17

The effect of its symbiont on the numbers of *Rh. trifolii* was noted during the year following clover (1932), the crop being ploughed under that spring preparatory to sowing timothy. The numbers remained more or less constant the second and third years (1933 and 1934) though in the spring of 1934 clover was sown with oats. During that year the plants did not develop to more than two or three inches in height, and no noticeable effect was found on the average numbers of nodule organisms in the soil. Only with the spring and summer of 1935, when the clover crop developed, was the presence of the symbiont reflected by a general rise in numbers of *Rh. trifolii*.

Apart from the effect of the recurrent growth of clover on *Rh. trifolii*, the study did not indicate any significant effect of the crops upon the numbers of the nodule organisms studied, nor did the moisture content of the soil or the season of the year appear to be of significance. Previous studies by one of us (6) on the effect of frost on the general microflora of soil had shown that after more than three months' freezing bacterial numbers remained at normal levels. Data from the present work (see Table II) indicate that the legume nodule bacteria in the soils studied are to be regarded as cold tolerant, *i.e.*, able to withstand the prolonged period of continuous freezing with relatively little or no diminution in numbers. This ability to tolerate continued frost is believed to be an important factor in the maintenance of successful legume cultivation in regions with severe winters.

Azotobacter

The data from the individual *Azotobacter* determinations are shown graphically in Fig. 2, while summaries for the successive years and average monthly values are presented in Tables II and V respectively. It is noted that Soil N,

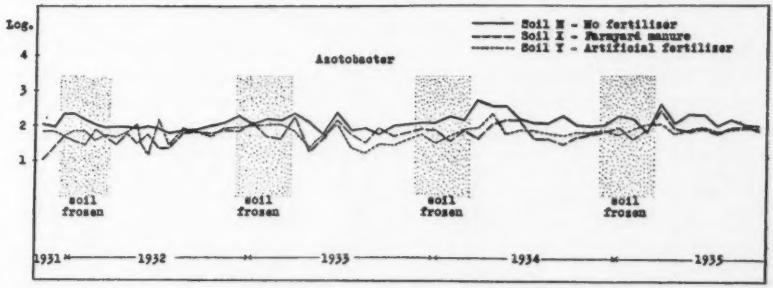

FIG. 2. *Numbers of Azotobacter in soils of different fertilizer treatment.*

without fertilizer for more than 20 years, and giving much lower crop yields than Soils X and Y, showed consistently the highest numbers of *Azotobacter*. The depressing effect of farmyard manure and nitrate fertilizer respectively on the numbers of *Azotobacter* is in agreement with the observations of Winogradsky and Ziemięcka (28), Batchelor and Curie (2), Curie (3), Gainey and Sewell (4), and Ziemięcka (29) who noted a reduction in the growth of *Azotobacter* from the application of nitrogenous fertilizers.

During the first year of the study, November, 1931, to October, 1932, parallel determinations were made of *Azotobacter* by the dilution method and of the nitrogen-fixing capacity of the soils in solution. The results, summarized in Table IV, show a general agreement between the dilution and the plate methods, though the absolute numbers found were higher with the former. Nitrogen fixation tests, however, indicated little or no difference between the soils, and are regarded as distinctly inferior to tests of the *Azotobacter* population, as determinable by silica-gel or agar plates and soil plaques in evaluating the *Azotobacter* flora of a series of soils.

TABLE IV

COMPARISON OF PLATE COUNT, DILUTION COUNT AND NITROGEN-FIXATION TESTS

Average, 16 determinations, November, 1931–October, 1932	Soil N (no fertilizer)	Soil X (manure)	Soil Y (mineral fertilizer)
Plate count, colonies per gram	100	37	59
Plate count, ratio	**100**	**37**	**59**
Dilution count, per gram	304	84	175
Dilution count, ratio	**100**	**28**	**58**
Nitrogen-fixation, mg. per 100 cc.	11.93	11.78	12.80
Nitrogen-fixation, ratio	**100**	**99**	**107**

A certain seasonal fluctuation in numbers of *Azotobacter* may be observed from Table V, giving the four-year monthly averages from the plate counts. In all soils, minimum numbers were found in July, in line with results reported by Vandecaveye and Anderson (16). Maximum numbers, however, were in

TABLE V

Azotobacter COUNTS—MONTHLY AVERAGES, 4 YEARS

Month	Colonies per gram dry soil		
	Soil N (no fertilizer)	Soil X (manure)	Soil Y (mineral fertilizer)
January	148	75	63
February	127	49	77
March	247 (max.)	99 (max.)	80 (max.)
April	145	56	62
May	133	58	69
June	133	72	69
July	80 (min.)	41 (min.)	41 (min.)
August	97	51	51
September	93	66	57
October	98	55	53
November	108	63	72
December	135	52	66

all cases found in March, in which month samples were all taken in the latter half, after the first thawing had occurred. This rise in numbers immediately following the period of frost was noted by one of us (6) in a study of the general bacterial soil flora. The data do not indicate any depressing effect of cold, however, the average counts being well maintained during the winter months.

One of the most striking features of the present study, as well as those of other workers, is the generally low number of *Azotobacter* in soils as indicated by any cultural method of enumeration. When one finds a few hundreds or at most a few thousands in a gram of soil which may harbor a bacterial population of one hundred million in addition to numerous fungi, protozoa, etc., one may reasonably doubt the importance of *Azotobacter* as an agent of nitrogen fixation under field conditions. The lack of exact knowledge of the role of this organism in the field has been emphasized by such workers as Waksman (17) and Winogradsky (27). Data from physiological studies of *Azotobacter* in pure culture, and from nitrogen-fixing capacity tests under artificial conditions do not appear to provide an answer to the question.

It is possible that our counting methods do not give a reasonable estimate of *Azotobacter* numbers in soil, since microscopic observations suggest, though they do not prove, a higher population. Thus Smith (10) found strains of *Azotobacter* which do not utilize mannite and would therefore be missed with media depending upon that carbohydrate, while van Niel (15) found failure of certain soils to give *Azotobacter* growth in an elective culture medium to be due to deficiency of molybdenum. Control tests did not indicate that these particular factors were operating in the present experiment. The recently

reported findings of filterable forms of *Azotobacter* in soil by Norogrudsky and Messineva (8, 9) suggest further the possible presence of this organism in numbers not indicated by present counting methods. Further progress in methods for determining the real *Azotobacter* population of soils would greatly help in forming an estimate of the actual role played by the organism in nitrogen fixation in field soils. Furthermore, the presence in soil of numerous other aerobic organisms to which capacity for nitrogen fixation has been ascribed, and more particularly, the possible importance of anaerobic bacteria as agents of nitrogen fixation under practical conditions confirm the belief that the part played by *Azotobacter* is an open question.

Total Bacteria Counts

Data from the total counts of bacteria and actinomyces, seen in Fig. 3 and Table II, do not indicate marked differences between the soils studied. Somewhat higher average counts were obtained from the two fertilized areas,

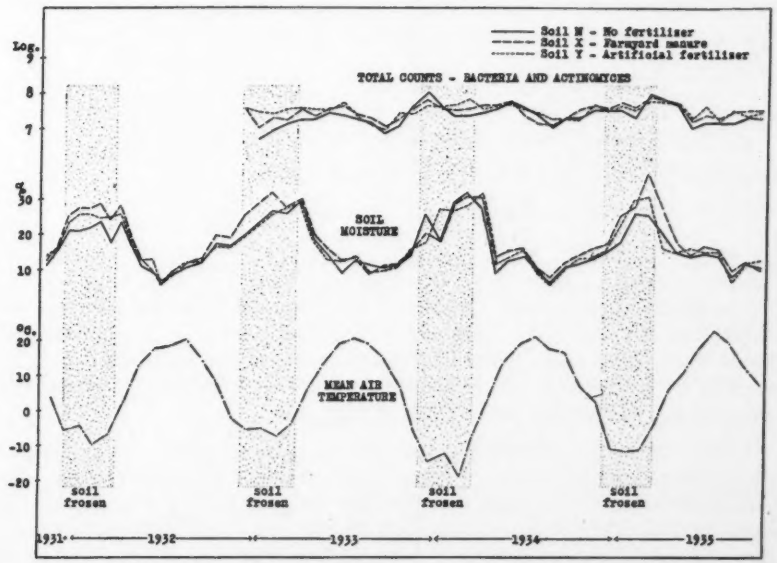

FIG. 3. *Total counts of bacteria and actinomyces, soil moisture and air temperature.*

X and Y, than from the unfertilized area, with numbers well maintained when the soil was frozen. As noted in other investigations with Canadian soils (5, 6, 7, 13), a certain seasonal trend was apparent, with generally lower numbers from July to September following higher numbers in spring. The data indicate, however, that for estimating the relative abundance of specific physiological groups of organisms in soil, such as nitrogen-fixing types, the total count method is unreliable. Its application to problems of soil fertility, then, must be considered as very limited.

Micro-organisms and Crop Yields

A comparison between the relative productivity of the three areas studied, and the average values for the bacterial counts are given in Table VI. Relative average yields for the different crops during the period of the test are given as well as relative counts (logarithms) for the various groups of micro-organisms.

TABLE VI

RELATIVE YIELDS AND NUMBERS OF BACTERIA

Crop	Soil area	Relative values					
		Crop yield	*Rh. trifolii*	*Rh. leguminosarum*	*Rh. meliloti*	*Azotobacter*	Total count
Timothy	N	100	100	100	100	100	—
	X	164	109	161	153	77	—
	Y	134	99	167	142	87	—
Mangels	N	100	100	100	100	100	100
	X	1,070	100	157	140	87	103
	Y	950	95	156	135	85	104
Oats	N	100	100	100	100	100	100
	X	138	106	152	152	81	100
	Y	129	104	159	149	83	101
Clover	N	100	100	100	100	100	100
	X	239	104	163	142	90	102
	Y	185	102	170	139	88	101

The relative productivity varies with the crop, the difference between the unfertilized and fertilized areas being most emphasized with mangels and less with the other crops. Although this pronounced difference with the hoed crop is not reflected in the bacterial counts, yet the relation of productivity to micro-population is of interest.

With total count, *Azotobacter* count, and *Rh. trifolii* count, no relation to productivity can be noted. The numbers of *Rh. leguminosarum* and *Rh. meliloti* show distinctly higher values when the fertilized areas are compared with the less productive area, N. Only in the case of *Rh. meliloti*, however, do the numbers found coincide in order with the order of productivity of all three soils for all four crops. It is suggested from the findings that study of the abundance of certain species shows greater promise of providing a reliable index of soil fertility than the numbers of micro-organisms as a whole.

Acknowledgment

The authors are indebted to the Field Husbandry Division, Central Experimental Farm, for co-operation throughout the course of the experiment and to the Division of Chemistry for the nitrogen determinations.

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THERMAL DEATH POINT OF CERTAIN WEED SEEDS[1]

By Clarence Yardley Hopkins[2]

Abstract

A series of weed seeds comprising seven species and representing five families was studied to determine the effect of heat on subsequent germination. Lethal temperatures for 15 minutes' heating varied from 85° to 105 ° C.

The results indicate that there is a critical temperature below which moderate periods of heating have little effect on viability. At higher temperatures the germinating power falls off rapidly.

Introduction

During a study of the utilization of refuse grain screenings, it occurred to the writer that a simple process for killing the weed seeds in screenings would be of practical value.

It is well known that the application of sufficient heat will destroy the germinating power of seeds. The effective temperatures and duration of heating have been determined for a number of seeds of crop plants. The present work was undertaken in order to determine the corresponding lethal conditions for some of the most common weed seeds found in refuse screenings.

A careful study of the effect of heat on radish seeds was made by Waggoner in 1917 (6). Other investigators have worked with cotton seed (3, 4) and with wheat (1, 8). The subject is reviewed and a bibliography appended in references 6 and 8.

It has been shown by these authors that the moisture content of the seed during the period of heating is an important factor in determining the lethal temperature. This condition depends not only on the initial moisture content of the seed, but also on the design of the heating chamber, *i.e.*, whether or not it allows free escape of moisture. Seeds which are very dry or are exposed to the drying effect of much warm air during the treatment will resist unusually high temperatures.

Experimental

Materials and Methods

The method of heat-treating the seeds was designed with the above considerations in mind. Furthermore, the conditions of the experiments were such that they could be easily duplicated in large-scale operation.

Most of the seeds were obtained from the weed nursery at the University of Saskatchewan through the kindness of T. K. Pavlychenko. A few samples were selected from refuse screenings taken from grain at Fort William.

The seeds were conditioned at 50% relative humidity at 25° C., so that all were comparable with respect to moisture content. The heating was carried out in narrow brass tubes, closed at the lower end and stoppered with a plug

[1] *Manuscript received February 15, 1936.*
Contribution from the Division of Chemistry, National Research Laboratories, Ottawa, Canada.

[2] *Chemist.*

of absorbent cotton. The tubes were immersed nearly to the top in a well-stirred oil bath with thermostatic control. Temperature readings were taken from a thermometer inside a similar tube. The seeds were treated in lots of such a size that the tube was filled to a depth of about one inch. The sample was then about two inches below the surface level of the bath. Following the heat treatment, the sample was poured into a bottle and allowed to cool before the bottle was stoppered.

Germination tests were carried out at the laboratory of the Seed Branch, Dominion Department of Agriculture, through the courtesy of the staff of that department.

Experimental Results

The relation of the temperature of heating to subsequent germination of some of the common weed seeds is shown in Figs. 1 and 2. The period of heating in each case was 15 minutes.

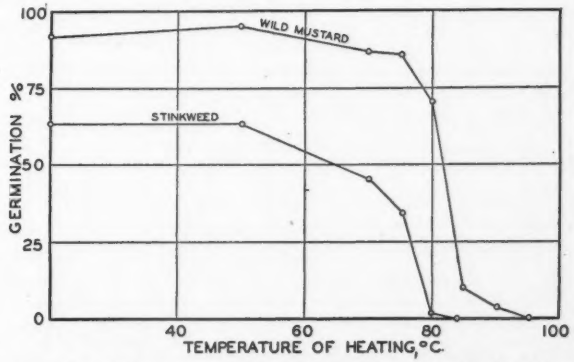

FIG. 1. *Effect of 15 minutes' heating on subsequent germination (Cruciferae).*

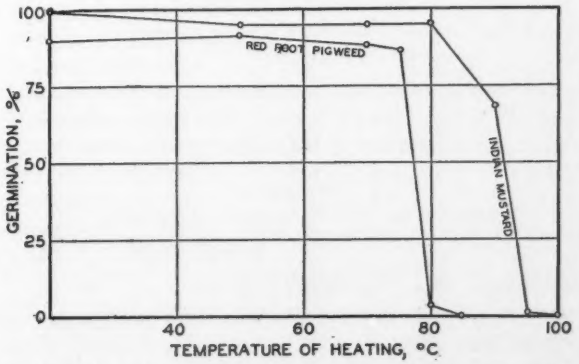

FIG. 2. *Effect of 15 minutes' heating on subsequent germination. (Cruciferae and Amaranthaceae).*

Figs. 3 and 4 show the temperature-germination curves for lambs' quarters and wild oats along with some seeds of other families for comparison. The period of heating was 15 minutes.

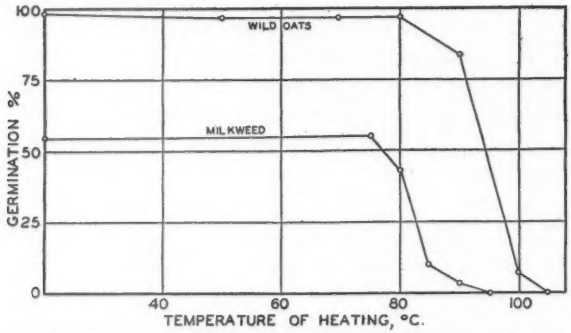

FIG. 3. *Effect of 15 minutes' heating on subsequent germination. (Graminae and Asclepiadaceae).*

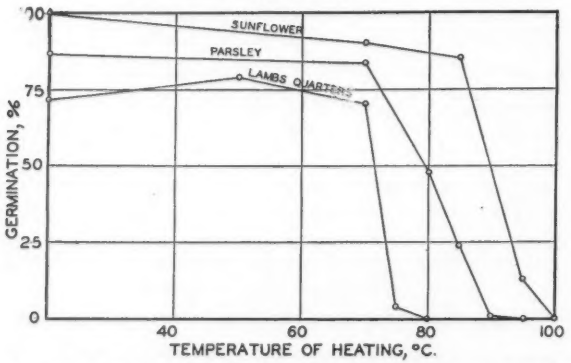

FIG. 4. *Effect of 15 minutes' heating on subsequent germination. (Compositæ, Umbelliferae and Chenopodiaceae).*

The effect of varying the time of heating was studied with seeds of Indian mustard, as shown in Table I. It is evident that increasing the time of heating has little effect on subsequent germination except at comparatively high temperatures.

TABLE I

GERMINATION OF INDIAN MUSTARD AFTER HEATING

Temp. of heating	Germination, %				
	70° C.	80° C.	85° C.	90° C.	95° C.
Period					
10 min.	96	92	89	87	—
20 min.	94	89	85	73	1
30 min.	97	94	89	56	—
60 min.	96	96	79	45	—

The approximate lethal temperatures under the conditions of the experiments are obtained by interpolation from Figs. 1–4 and are shown in Table II. These temperatures refer to seeds whose moisture content is such that they are in equilibrium with air at 25° C. and 50% relative humidity. The percentage moisture in the seed under these conditions will depend on the species, starchy seeds having a greater affinity for water than fatty seeds.

TABLE II

LETHAL TEMPERATURES

(15 minutes' heating)

Species	Initial germination, %	Lethal temperature, °C.
Indian mustard (*Brassica juncea*)	99	100
Wild oats (*Avena fatua* L.)	98	105
Wild mustard (*Brassica arvensis* (L)-Ktze)	91	95
Red root pigweed (*Amaranthus retroflexus* L.)	90	85
Lambs' quarters (*Chenopodium album* L.)	72	95
Stinkweed (*Thlaspi arvense* L.)	63	85
Milkweed (*Asclepias syriaca* L.)	54	95
Sunflower (*Helianthus annuus* L.)	100	100
Parsley (*Carum petroselinum* B & H.)	86	90–95

In addition to the species listed in Table II, lethal temperatures for fifteen minutes' heating were determined approximately for the following seeds: hare's ear mustard (*Conringia orientalis* (L) Dumort) 80° C.; ball mustard (*Neslia paniculata* (L) Desv.) 85° C.; Russian pigweed (*Axyris amaranthoides* L.) 80° C. The initial germination of these samples, however, was not as high as that of the other seeds described above.

The moisture content of wild mustard seed, when kept at 25° C. and under four different conditions of relative humidity, was determined. The results are compared with similar observations on wheat reported by Hottes and Wilson (1).

TABLE III

MOISTURE CONTENT OF SEEDS UNDER DIFFERENT CONDITIONS OF HUMIDITY

Relative humidity	Moisture content, %	
	Wild mustard, 25° C.	Wheat, 25° C.*
30%	5.38	—
50	6.57	9.8
60	7.18	14.4
70	7.78	ca.19

**Data from Hottes and Wilson (1).*

Discussion

The results show that in order to destroy the viability of a mixture of all the weed seeds tested, it would be necessary to use a temperature of slightly above 100° C. under the conditions of these experiments. Wild oats, the most resistant, showed 7% germination after 15 minutes' heating at 100° C.

but no germination after heating at 105° C. A temperature of 100° C. might be sufficient if the period of heating were increased to 20 minutes.

In some cases, a slight increase in *total* germination was noted after moderate heating (50° C.). Other investigators have observed an increase in the *rate* of germination subsequent to mild heat treatment.

It may be noted from the curves in Figs. 1–4 that the viability diminishes rapidly as the temperature of heating approaches the lethal point. This suggests that there is a critical temperature for each species below which a short period of heating has no appreciable effect on subsequent germination while higher temperatures cause rapid destruction of germinating power. The same effect is observed in earlier studies on corn, wheat, and tomato seeds, although Waggoner found a more gradual diminution in viability of radish seeds. These results are compared in Table IV with some of the results of the present work.

TABLE IV

EFFECT OF HIGH TEMPERATURES ON VIABILITY OF SEEDS

(Including data from earlier studies)

Temp. of heating, °C.		Germination, %										
		62.5	65	67.5	70	75	80	85	90	95	100	105
Corn	*(a)*	84	36	2	0							
Radish	*(b)*		91		83	74	64	56	28	0		
Tomato	*(c)*						95	38	3	0		
Wheat	*(d)*				96		100		8		0	
Red root pigweed	*(e)*				88	86	4	0				
Wild oats	*(e)*				95		96		84		7	0

(a) Moisture 12.5%. Heated 2 hours (5)
(b) Moisture 9%. Heated 30 minutes (6).
(c) Heated in air oven, 3 hours, (2).
(d) Moisture 9.9%. Heated 30 minutes (1).
(e) Present investigation. Heated 15 minutes.

The mechanism of the loss of viability on heating is unknown. Robbins and Petsch (5) studied the rate of coagulation of egg albumen at various temperatures in conjunction with their experiments on wheat and corn and observed some correlation between the rate of coagulation of albumen and the loss of germinating power in the seeds. It is inferred that heat coagulates the reserve protein of the seed and renders it incapable of being utilized.

The possibility exists that the loss of viability is more directly due to inactivation of the enzymes by heat. The resistance of enzyme preparations to heating at various temperatures is closely parallel to the behavior of seeds under the same conditions. According to Waksman (7), most enzymes are rapidly destroyed by heating in water at 70–80° C. When dried they will withstand temperatures of 100–120° C. or higher. Seeds treated under these conditions exhibit the same behavior with respect to viability.

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STUDIES ON THE ENDOPARASITIC FAUNA OF TRINIDAD

III. SOME PARASITES OF TRINIDAD CARNIVORES[1]

BY THOMAS W. M. CAMERON[2]

Abstract

Two new species of cestodes, five of nematodes, two of Acanthocephala and one linguatid are recorded from carnivores in Trinidad, B.W.I.

During 1935, the writer received from Professor F. W. Urich, Trinidad, B.W.I., the entrails of an ocelot or tiger-cat *(Felis pardalis)*, four "mangrove dogs" or crab-eating racoons *(Procyon cancrivora)* and three "wood-dogs" or Tayras *(Tayra barbara)*. The only other carnivore known to exist in Trinidad, is the very rare otter *(Lutra insularis)*, and so far it has not been possible to secure a specimen. All of the eight animals received contained internal parasites. These consisted of two species of cestodes, five of nematodes, two of acanthocephala and one linguatid; no trematodes were found.

In addition to parasites, the stomachs and intestines of the racoons contained many remains of beetles and crabs, while in the ocelot were parts of an agouti as well as two decomposed trichostrongyles of a species found in that host: these accordingly, are not recorded as parasites of the ocelot.

I have to acknowledge the assistance both of Professor R. T. Leiper of the Imperial Bureau of Agricultural Parasitology and Dr. M. C. Hall of the United States Bureau of Animal Industry, in obtaining both references and literature. I have also to acknowledge the willing assistance of the Division of Research Information of the National Research Council of Canada in the translation of a number of papers.

Cestoda

Two—possibly three—species of cestodes, all belonging to the genus *Diphyllobothrium*, were recovered from the intestines of Trinidad carnivores. One species was found in the ocelot, and one in the racoon: the third species was also found in the racoon in very large numbers and may merely represent the young stages of the second species. In order, however, to avoid subsequent confusion, it is described merely as *Diphyllobothrium* sp.

[1] *Manuscript received March 20, 1936.*
Contribution from the Institute of Parasitology, McGill University, Macdonald College, P.Q., with financial assistance from the National Research Council of Canada.

[2] *Research Professor of Parasitology, McGill University; and Director, Institute of Parasitology, Macdonald College, P.Q., Canada.*

Diphyllobothrium urichi sp. nov.

Several specimens of this species were recovered from the small intestine of the ocelot *(Felis pardalis)*. They had been fixed *in situ* in formalin, and the process of opening the intestine unfortunately prevented any complete tapeworms being recovered. The total length cannot, therefore, be given, but several pieces more than 20 cm. long were recovered, and it is probable that specimens reached at least 30 cm. in length.

A single head was seen (Fig. 1.). It was almost as long as it was broad. The bothria are conspicuous and have free anterior margins; the posterior margins gradually join the neck. The anterior end of the scolex projects beyond the bothria, which appear to be situated laterally; owing to twisting of the neck, this point requires confirmation. The neck is long but broad and the whole appearance of the anterior end of the body is quite different from that of *D. latum*.

The mature segments are shown in Figs. 2 and 3, the latter being an older one towards the end of the chain. The oldest segments are approximately square: the younger ones are broader than long. None is longer than broad.

The male and female openings are on the mid-ventral line, almost a quarter of the length of the segment from its anterior margin. The uterine pore is almost at the centre point of the segment but is always slightly to one side of the mid-ventral line.

The uterus is convoluted, being thrown into four or five small lateral loops. It passes for a short distance in front of the uterine pore but shows no tendency to form a "rosette." It always passes posterior to the ovarian commissure and, in fully mature segments, always makes an indentation in the posterior margin.

The ovary is quite typical. The yolk follicules and testes are very numerous and almost equal in size. They completely fill both sides of the segment and meet in the middle line, anterior to the genital pore. Both testes and yolk follicules are completely absent from an oval space around the genital tubes and they do not meet posterior to the ovary.

The eggs are oval and measure 50μ long by 30μ broad.

In this species, the uterus is confined to the medial region of the posterior half of the segment, and a post-ovarian portion, in fully mature segments, always projects into the succeeding segments. The genital pore opens about a third of the distance from the anterior border and both testes and yolk glands are continuous across the middle line, anterior to the cirrus sac, but *not* posterior to the ovary.

Its closest relation would appear to be *D. decipiens* (Diesing) as described by Faust, Campbell and Kellogg (2) from Asia. It differs, *inter alia*, in the posterior portion of the uterus, which in *D. decipiens*, is not pushed backwards into the next proglottid.

D. decipiens has been recorded from Uruguay by Wolffhügel and Vogelsang (16) on the basis of feeding experiments with a plerocercoid which they found in *Didelphis paraguayensis*. A study of the adult cestode convinced them it was *D. decipiens* (Diesing, 1850) (Syn. *D. serratus* Diesing 1850), and they point out that it differs from *D. latus* in its smaller size and absence of a uterine rosette. However, recent work has shown that the rosette shape of the uterus is seen in very few species and that its absence is a sub-generic rather than a specific character (2). Wolffhügel and Vogelsang (16) found the same species in a jaguar *(Felis onca)* and they state that it has also been recorded from Brazil in *Felis yaguarundi* by Parodi and Widakowich under the name of *D. longicollis*.

If Wolffhügel and Vogelsang's species is the same as that described by Faust, Campbell and Kellogg, then the present tapeworm is not the same species as that found in the south of South America.

D. reptans (Diesing, 1850), has also been recorded from South America, occurring as a larva in this host as well as in other carnivores, including *Tayra barbara*. There is some doubt as to the identity of *D. reptans*. Some workers have considered it is identical with *D. ranarum*. If this opinion is correct, the present species definitely differs from it. The whole genus is very imperfectly known and it is regarded as preferable to consider the present form as a new and distinct species, of which the most outstanding character is the shape and disposition of the uterus; accordingly, the name *D. urichi* sp. nov. is proposed for it.

Diphyllobothrium trinitatis sp. nov.

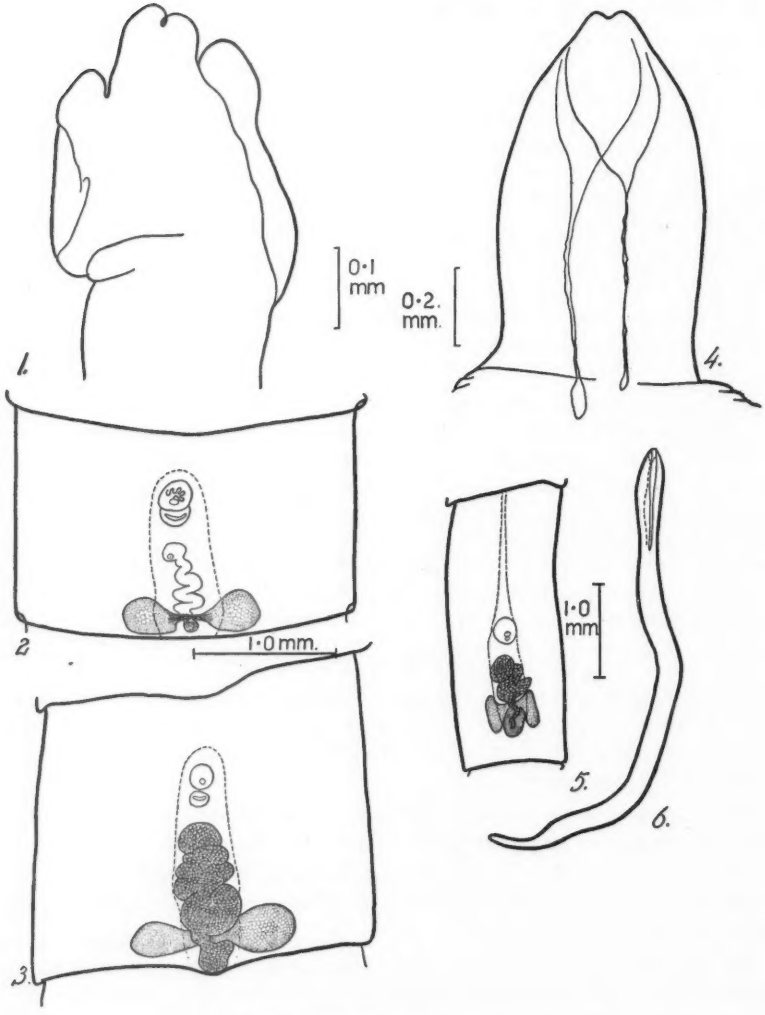
A large number of a second species of this genus of cestodes was recovered from the small intestine of a crab-eating racoon *(Procyon cancrivora)*. They were accompanied by a number of obviously recently ingested plerocercoids which are discussed later.

The adult worms are much smaller than the forms found in the ocelot, and none was more than 10 cm. in length.

The scolex (Fig. 4) has large bothria on its dorsal and lateral surfaces, and is bluntly conical in shape. There is no neck, segmentation beginning immediately at the end of the bothria.

The segments at first are very short and wide but at the posterior end of the chain they are nearly three times as long as broad (Fig. 5).

The genital openings are almost in the exact centre of the ventral surface of the segments; the uterine opening is a short distance posterior to this and is almost medial. The uterus is towards the posterior end of the segment and has fewer loops than the previous species: moreover, unlike it, these loops are dorso-ventral rather than lateral. The uterus never extends posterior to the ovary, which is situated a short distance from the posterior margin of the segment.



Diphyllobothrium urichi sp. nov.

FIG. 1. *Scolex.* FIG. 3. *Old mature proglottid.*

FIG. 2. *Young mature proglottid.*

Diphyllobothrium trinitatis sp. nov.

FIG. 4. *Scolex.* FIG. 5. *Mature proglottid.*

Diphyllobothrium sp. inq.

FIG. 6. *Entire parasite.*

N.B.—*In Figures 2, 3 and 5, the testes and yolk glands are* not *shown, the broken line in each case representing their medial limits.*

Both testes and yolk glands are numerous and, as usual, occupy the two lateral fields. Both meet in the mid-line posterior to the ovary. Anterior to the genital openings, however, the testes alone meet in the mid-line, the two groups of yolk glands having a small space between them which extends to the anterior limit of the segment.

The eggs are oval and, while about the same width as those of the previous species, they are a little longer (58μ by 28–30μ) giving them a much more elongated appearance.

This species differs markedly from all other described species and it is, in consequence, regarded as new, under the name of *Diphyllobothrium trinitatis*. It may be distinguished by the shape of the head, absence of neck, disposition of uterine tube, and the relative distribution of testes and yolk glands.

Diphyllobothrium sp. inq.

In addition to *D. trinitatis* a considerable number of young forms were recovered from the same host. These (Fig. 6) consist only of a scolex and neck and probably represent recently ingested plerocercoids. The shape of the scolex is of the *D. latum* type and consequently, it has been considered desirable to regard it as a *species inquerenda* rather than refer it to any known form. It may, of course, be the young stage of *D. trinitatis*.

Nematoda

Necator urichi sp. nov.

Four females but no males, of this species were recovered from a racoon *(Procyon cancrivora)*. The length varies from 4.5 mm. to 6 mm. The buccal capsule (Figs. 7 and 8) is of the typical *Necator* type. Viewed from the side it is almost triangular in outline, and the mouth opening is directed in a definite dorsal direction. It is guarded by two obvious "cutting-plates." On the ventral wall of the buccal capsule are two small, triangular lancets, and on the dorsal wall, a single, conspicuous dorsal cone.

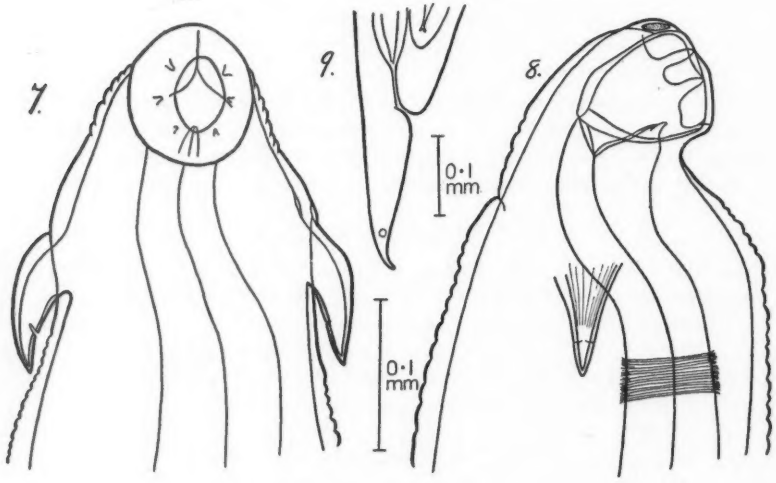
The oesophagus is of the usual type, measuring 0.45 mm. in length and 0.1 mm. at its thickest point.

The excretory pore is situated anteriorly, not far distant from the base of the buccal capsule, while the nerve ring encircles the oesophagus, about 0.15 mm. from its anterior end.

Midway between these two points are two very large cervical papillae—one on each lateral line. They are massive triangular structures which seem like appendages rather than papillae. They are identical on all four specimens so that they cannot be considered as abnormalities. They are sharply pointed, and a short distance from the apex are *two* nerve endings, contained in smali depressions, one on the medial aspect of each margin. While the appendages are in all cases held against the sides of the worms, they contain many strands of tissue resembling muscle fibres, suggesting that in life they are movable.

The cephalic and cervical glands are conspicuous and measure about twice the length of the oesophagus.

The ovarian coils are mainly lateral and the vulva is situated just anterior to the middle of the body, which it divides in the ratio of 7 : 8.



Necator urichi sp. nov.

FIG. 7. *Dorsal view of head end.* FIG. 9. *Lateral view of tail of female.*
FIG. 8. *Lateral view of head end.*

The anus is a transverse slit, situated 0.2 mm. from the tip of the sharply pointed tail. Towards the tip of the tail are two small sessile caudal papillae (Fig. 9).

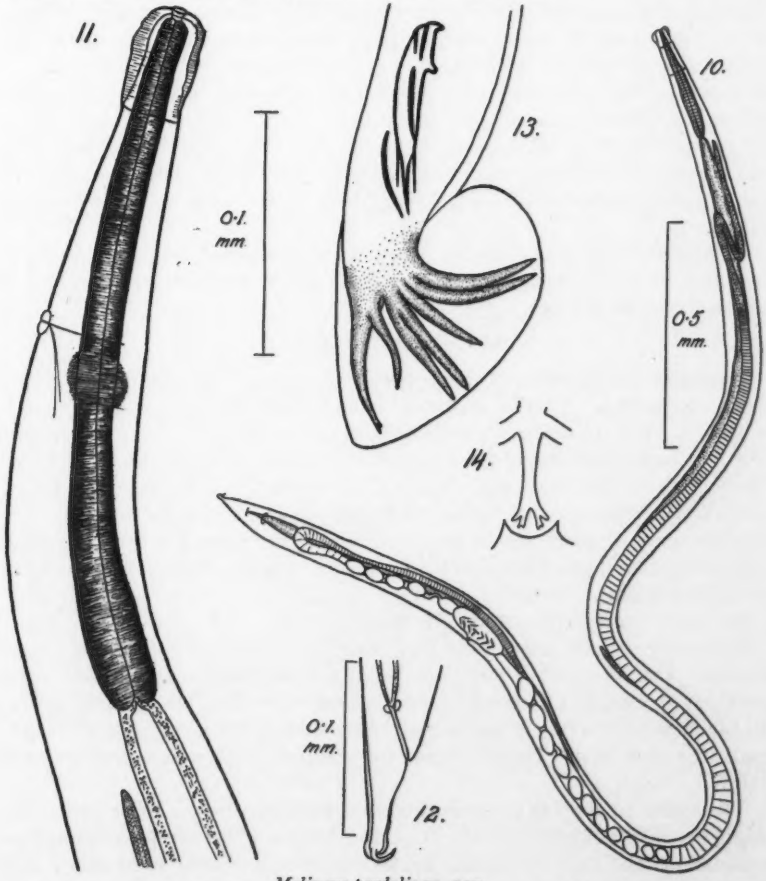
In the absence of males, this species must be referred to the genus *Necator*, although subsequent work may show that it should be removed to a new genus.

Two species of hookworms have been previously recorded from this host in continental South America by Molin (4) in 1860, *viz.*, *Dochmius bidens* and *D. maxillaris*. Professor Leiper has kindly loaned me sketches which he had made from Molin's original material of these two species. These two sketches and Molin's original descriptions show quite clearly that neither is this species. In both, for example, the dorsal cones are absent. The only other hookworm recorded from South American carnivores is *Uncinaria carinii* Travassos (9) in 1915 from *Canis azarae*. Travassos himself draws attention to the fact that this last species is very similar to *U. criniformis* (Goeze, 1782), which also has no dorsal cone. The present species, which is characterized not only by the possession of a dorsal cone, but by the presence of enormously enlarged cervical papillae (amounting to appendages) is accordingly regarded as new and is named *Necator urichi* sp. nov.

Molineus pardalis sp. nov.

Two females and five males of this species were recovered from the small intestine of the ocelot *(Felis pardalis)*.

The females (Fig. 10) have an average length of 3.6 mm. and the males 2.7 mm. The head end is swollen (Fig. 11) and finely transversely striated. There are about 16 main and numerous fine longitudinal striations on the body. The oesophagus is simple and only slightly swollen posteriorly; it is about 0.3 mm. long. The nerve ring surrounds it about its middle point. Cervical papillae are absent. The excretory pore is conspicuous and is situated just anterior to the nerve ring; it lies on a groove (which, however, disappears on the dorsal surface) and is protected by two small lips.



Molineus pardalis sp. nov.

FIG. 10. *Entire female.*
FIG. 11. *Head end, lateral view.*
FIG. 12. *Tail of female.*
FIG. 13. *Bursa of male, lateral view.*
FIG. 14. *Dorsal ray of bursa (to same scale as 13).*

The female genital system is double and simple. The uteri are opposed and terminate in conspicuous ovejectors. Each uterus contains four or five eggs in a single row. The superior ovary is almost straight, originating about the base of the oesophagus. The inferior ovary turns on itself just in front of the rectum; its origin is in the middle of the body. The vulva is 0.65 mm. and the anus, 0.05 mm. from the end of the body, which is slightly swollen (Fig. 12) and then projects as a sharp, simple spine.

The males (Figs. 13 and 14) have a simple bursa, which is continuous ventrally. There are two large lateral lobes and a single small dorsal lobe. The ventral rays are equal and parallel and widely separated from the others. The externo-lateral ray is shorter than the other laterals and does not reach the edge of the bursa. The externo-dorsal rays originate almost at the base of the main dorsal stem and are parallel to the lateral group. The dorsal ray bifurcates near its tip (Fig. 14) to form a long-stemmed Y. Each bifurcation divides into two approximately equal parts, the medial of which are divided terminally. Pre-bursal papillae are absent.

The spicules (Fig. 13) are similar and equal. They are short stout structures, about 0.075 mm. long, terminating in three short sharp spines. The gubernaculum, although about half as long as the spicule, is slender and curved.

This species is smaller than the type species, *M. felineus* Cameron, 1923 (1). It differs from it especially in the relatively large externo-lateral ray and in the shape of the spicules.

Molineus major sp. nov.

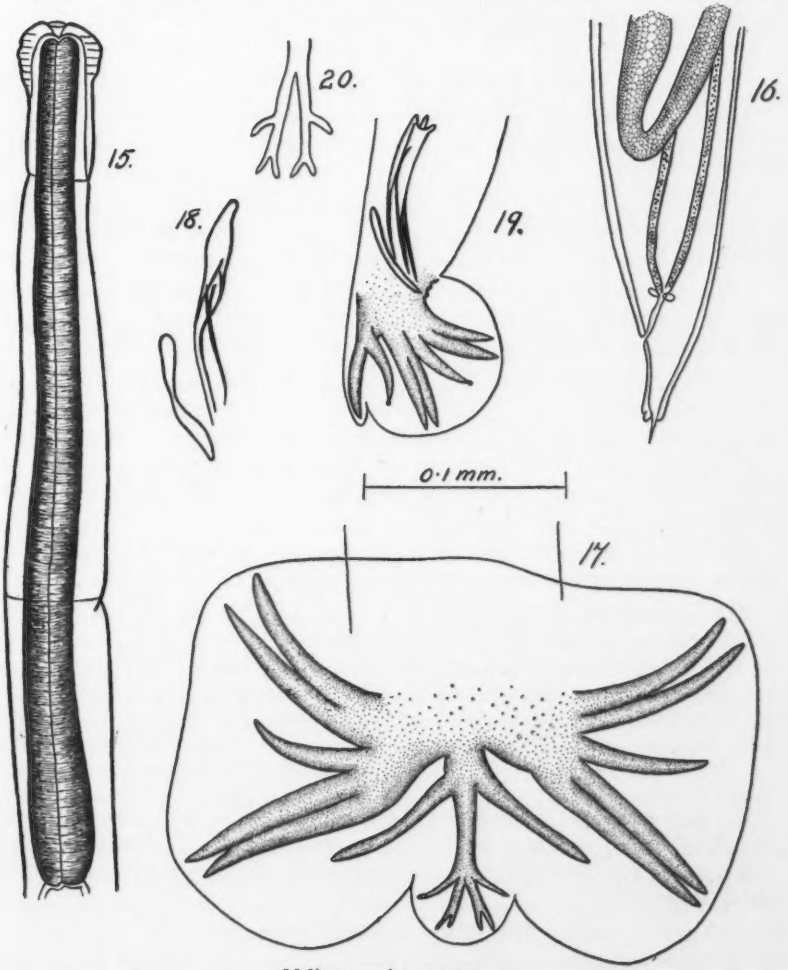
A number of specimens of this species were found in the small intestines of three "wood-dogs" *(Tayra barbara)*. The females measure 8.75 to 9 mm. in length, while the males are little more than half this size, *i.e.*, 5 to 5.5 mm. The maximum thickness of the females is 0.15 mm. and of the males 0.09 mm. The cuticle of the head end (Fig. 15) is swollen, but unlike the previous species, it is conspicuously transversely striated in its anterior portion. The anterior portion moreover is much more swollen than the posterior part. While longitudinal striations are conspicuous in the rest of the body, transverse striations are also present.

Six small circum-oral papillae are present.

The oesophagus is about 0.45 mm. long in the male and 0.55 mm. in the female. The lips of the excretory pore are less conspicuous than in the previous species, but a complete ring is present in the cuticle at this point. In the females this ring is just behind the middle of the oesophagus but in the male it is even farther back, lying at the junction of the middle and posterior thirds.

The vulva in the female is about 1.7 mm. from the posterior end. The vagina is more powerful and the ovejectors longer. The uteri are much more voluminous and each contains a *double* row of eggs—some 50 in each. The anus is about 0.05 mm. from the tail, which is swollen—often with small secondary swellings. The spine is quite conspicuous (Fig. 16).

The bursa in the male is large and rectangular (Fig. 17), and the dorsal lobe is distinct and relatively large. The bursal formula is very similar to that seen in the type species, but the externo-lateral rays are rather longer and more divergent. The dorsal ray splits into four digitations of almost equal length; the inner, however, are more massive and are divided at their tips.



Molineus major sp. nov.

FIG. 15. *Head end.*
FIG. 16. *Tail of female.*
FIG. 17. *Bursa of male, ventral view.*
FIG. 18. *Spicules and accessory piece, side view.*

Molineus barbaris sp. nov.

FIG. 19. *Bursa and spicules of male, side view.* FIG. 20. *Diagram of dorsal ray.*
N.B.—*All drawings, except Fig. 20, are to the same scale.*

The spicules (Fig. 18) which measure 0.13 mm. in length, are very similar to those seen in *M. felineus*. They are very faintly cuticularized. The gubernaculum is about 0.075 mm. long.

This species can be separated from the type species by the points mentioned above, especially by the voluminous nature of the uteri. The name *Molineus major* sp. nov. is proposed for it.

Molineus barbaris sp. nov.

A few examples of a second species of *Molineus* were recovered from two of the wood-dogs. The fact that it was not present in the other, made the separation easy.

It is a much smaller species, the females being only 4.1 mm. and the males 3.4 mm. in length. The head is similar to the last species, but the excretory pore is conspicuous and the ring is anterior to the middle of the oesophagus.

The vulva is situated 0.85 mm. from the tail and the lips are conspicuous. The uteri are much smaller, carrying a single row of 10–12 eggs in each. The tail is similar to the last species, the anus being 0.075 mm. from the tip.

In the male, the dorsal lobe (Fig. 19) is comparatively conspicuous, although the whole bursa is small. The externo-lateral ray is comparatively large, although still smaller than the other laterals. The termination of the dorsal ray (Fig. 20) differs also: there are two relatively long bifurcations, each of which splits terminally; about half way down each bifurcation a curved lateral process is given off.

The spicules, under 0.1 mm. in length, are provided with three long sharp points. The gubernaculum is about half the length of the spicules.

The name *Molineus barbaris* sp. nov. is proposed for this species, which is closely related to the type. It differs from it in several points however—in the complete cervical groove, in the completely separated externo-lateral ray, and in the shape of the dorsal ray. It is easily separated from *M. major* by its much smaller size and from *M. pardalis* by the shape of the spicules and of the dorsal ray.

Molineus sp.

A single somewhat decomposed female of this genus was recovered from the small intestine of a racoon *(Procyon cancrivora)*. It is not possible to refer it to any species, although the shape of the uterus suggests that it may be *M. barbaris*.

The Genus *Molineus* Cameron.

In 1923, the writer described the type species of this genus, *M. felineus* from *Felis yaguarundi* from Brazil. In the original description a cervical groove was stated to be absent, but a subsequent examination of the material, which was not in a good state of preservation, showed that a partial groove was present on the ventral side of the body, and the generic concept must

be altered in consequence. *M. felineus* is the only trichostrongyle which has been previously described from South American carnivores and the four species may be distinguished as follows:

Host	*M. felineus*	*M. pardalis*	*M. major*	*M. barbaris*
	Felis yaguarundi	*Felis pardalis*	*Tayra barbara*	*Tayra barbara*
Length—female	5.25 mm.	3.6 mm.	8.75–9 mm.	4.1 mm.
Length—male	4.75 mm.	2.7 mm.	5–5.5 mm.	3.4 mm.
Transverse striations on cephalic swelling	Conspicuous	Fine	Conspicuous anteriorly	Conspicuous anteriorly
Excretory groove	Ventral only	Ventral only	Complete ring	Complete ring
Uterus:				
Eggs	In single row	In single row	In double row	In single row
Bursa:				
Externo-lateral	Half length other lateral rays	Three quarters length other lateral rays	Half length other lateral rays	Half length other lateral rays
	Separate from M.L. for half length only	Completely separate	Completely separate	Completely separate
Terminal digitation of dorsal ray	Conspicuous: lateral largest	Small	Conspicuous	Fairly conspicuous. Lateral small
Dorsal lobe	Indistinct	Indistinct	Distinct	Distinct

Physaloptera maxillaris Molin, 1860

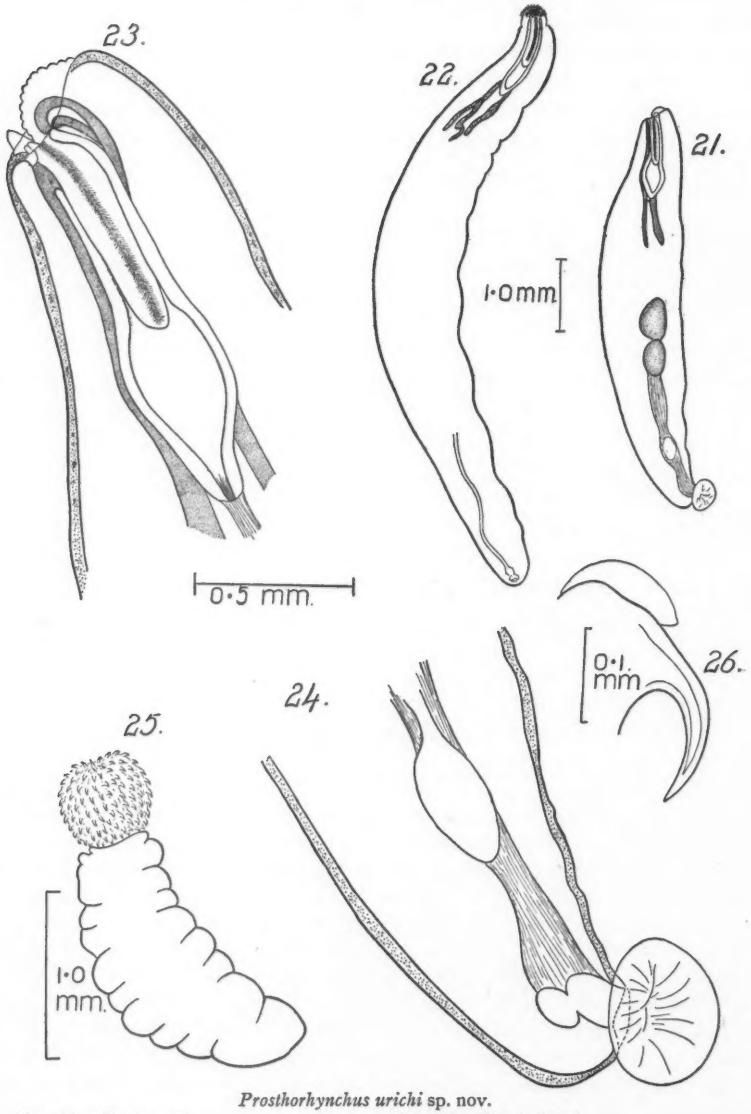
A single female of this species was recovered from the small intestine of *Procyon cancrivora*. It was fully developed but contained no eggs. It agrees in every respect with Ortlepp's (5) excellent description and the writer has nothing to add to it.

Acanthocephala

Prosthorhynchus urichi sp. nov.

One male and two female acanthocephalid worms were recovered from the small intestine of *Procyon cancrivora*. The male (Fig. 21) is 5.5 mm. long and the females (Fig. 22) about 8.0 mm. The cuticle is thick and smooth and is without spines. The "nuclei" of the sub-cuticula are branched. In all cases the proboscis is retracted, but in one female it is slightly extruded to show a very short neck, which is free from spines. The proboscis is long and cylindrical and is armed with numerous spines, which have long, sharp, recurved points and, so far as can be seen, single roots. The proboscis sheath is nearly cylindrical anteriorly, but is slightly swollen towards its posterior end (Fig. 23). It is inserted at the base of the proboscis, at the anterior end of the body: the proboscis should be completely extrusable. Three lemnisci are present; these are long and slender and about twice the length of the proboscis sheath.

The oval testes in the male lie tandem just behind the mid-point of the body, with their long axis in the direction of the body. The writer was unable to be certain of the details of the cement gland (prostate gland) in



Prosthorhynchus urichi sp. nov.

FIG. 21. *Entire male.*
FIG. 22. *Entire female.*
FIG. 23. *Head of male.*
FIG. 24. *Tail of male.*

Paridosentis sp.

FIG. 25. *Entire animal.*

Porrocephalus stilesi Sambon.

FIG. 26. *Outer hook.*

the single male, although it seems to form a single, elongated mass. A simple campanulate bursa is present (Fig. 24).

The two females are both immature and the egg masses were only in process of breaking down into eggs. One ovary lay just behind the proboscis sheath: a second could not be seen but a number of egg masses were scattered throughout the body cavity. The uterus is long and narrow, but the uterine bell could not be observed. The vaginal sphincter is small as is the vagina proper. The genital opening is sub-terminal.

These worms seem closely related to the genera *Plagiorhynchus* and *Prosthorhynchus*, none of the described species of which, however, possess a neck (7, 10, 11). Although unable to be certain of the structure of the cement glands (which is essential under our present criteria for the determination of genera of Acanthocephala), the writer is tentatively referring it to the genus *Prosthorhynchus*, pending the discovery of more mature specimens. As all the described species have so far been found in birds, it is possible that this also is a bird form in an abnormal host. Although some of the structures necessary for a correct conception of a species have not yet been seen, it is proposed to regard this form as a new species, *Prosthorhynchus urichi* sp. nov., with the following tentative diagnosis:— Body elongated, without spines, with short unarmed neck and long cylindrical proboscis which can be completely inverted and which carries numerous spines: three lemnisci: testes in middle region of body: cement gland elongated, but number of elements undescribed.

Paridosentis sp.

In addition to the forms described above, another acanthocephalid was recovered from the same host. This (Fig. 25) is obviously an immature form. It, however, closely resembles Van Cleave's description (13) of *Paridosentis iracundus* Van Cleave, 1920, from Venezuelan fish and it is provisonally referred to this genus, although absence of more mature forms prevents a specific determination.

Arthropoda

Porrocephalus stilesi Sambon, 1910

A single linguatulid was found in the small intestine of the ocelot *(Felis pardalis)*. It corresponds very closely to Sambon's description (6) of *Porrocephalus stilesi*, which occurs in the Bushmaster and Fer de Lance (both of these snakes are present in Trinidad, although I am unaware of any parasites having been recorded from them there). It is a young female, only 10 mm. long. There are about 50 annuli, and the tail, including the anal and female openings, closely resembles Sambon's drawing. The mouth is oval, but somewhat constricted anteriorly. The hooks are large, the inner pair being slightly larger than the outer. The straight accessory spine, described by Sambon as being present on the outer hooks, was not present: there was, however, a small piece of tissue present on each outer hook in the place where

the spine should be (Fig. 26). As the specimen is obviously a very young one, it is possible that this tissue represents the spine in the fully grown adult, which reaches a length of 80 to 96 mm.

The nymphal stages occur in mammals in other members of this genus, although they have not yet been described for this species and it is probable that this parasite is an accidental form in the ocelot. The same animal had, in its intestine, several decomposed trichostrongyles which are normally found in agouti, as well as remains of agouti in its stomach. The pentastome possibly came from the same source. Both snakes mentioned above are also known to feed on agouti.

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